PS-5, A NEW β-LACTAM ANTIBIOTIC. I
TAXONOMY OF THE PRODUCING ORGANISM, ISOLATION AND PHYSICO-CHEMICAL PROPERTIES*

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Antibiotic PS-5[1] is a new β-lactam antibiotic isolated from fermentation broths of *Streptomyces* sp. strain A271. The strain was considered to be a new subspecies of *Streptomyces cremeus* and the name, *Streptomyces cremeus* subsp. *auratilis*, was proposed. Fermentative production, isolation and physico-chemical properties of PS-5 are described.

During the course of screening for antibiotics produced by strains of Actinomycetes, several strains of *Streptomyces* were found to produce a new β-lactam antibiotic possessing strong antimicrobial activity against organisms resistant to known β-lactam antibiotics[2] and also having inhibitory activity against β-lactamases from *Bacillus cereus, Bacillus licheniformis*[3] and *Proteus vulgaris*[4]. One of the producing organisms, strain A271, was identified as a new subspecies of *Streptomyces cremeus*, and was named *Streptomyces cremeus* subsp. *auratilis*. In this paper, taxonomic studies of strain A271, fermentative production, isolation and physico-chemical properties of PS-5 are reported.

Taxonomic Studies of Strain A271

Strain A271 which produces the new β-lactam antibiotic PS-5 was isolated from soil collected near Eiheiji Temple at Eiheiji-cho in the Yoshida District of Fukui Prefecture in Japan.

Taxonomic studies were performed using principally the criteria recommended by SHIRLING and GOTTLIEB[5~9] as well as those reported by PRIDHAM and TRESNER[10] and WAKSMAN[11]. Reference strains employed in the studies were received from the Institute for Fermentation, Osaka.

Strain A271 shows the following characteristics:

Morphological Characteristics

Morphological features were observed after 1~3 weeks' incubation at 28°C on the various ISP (International Streptomyces Project[5]) media.

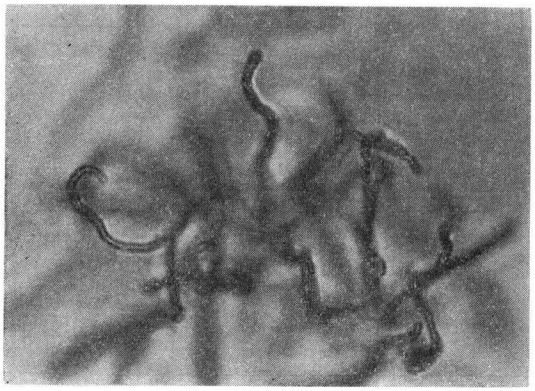
The aerial mycelium of strain A271 has dichotomous branches without verticils and the chains of spores form hooks, loops or incomplete spirals on oatmeal agar and glycerol-asparagine agar. Straight or flexuous forms are ocassionally observed on yeast extract-malt extract agar (Plate 1).

The spores forming chains of 10~50 are oval or cylindrical and 0.8~1.0×1.0~1.8 μ in size.

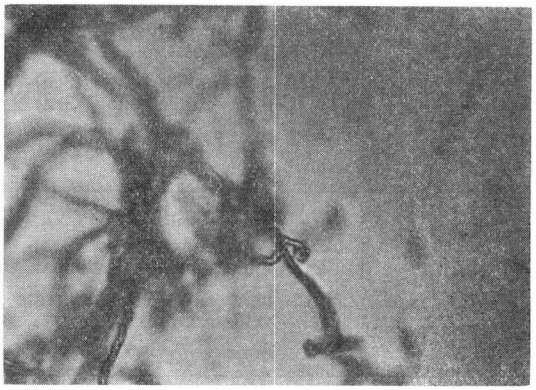
* Presented at the 209th Scientific Meeting of Japan Antibiotics Research Association, May 29, 1978 (Tokyo).

Plate 1. Aerial mycelium of strain A271. Culture: 28°C, 14 days.

(a) Oatmeal agar



(b) Glycerol-asparagine agar



(c) Yeast extract-malt extract agar

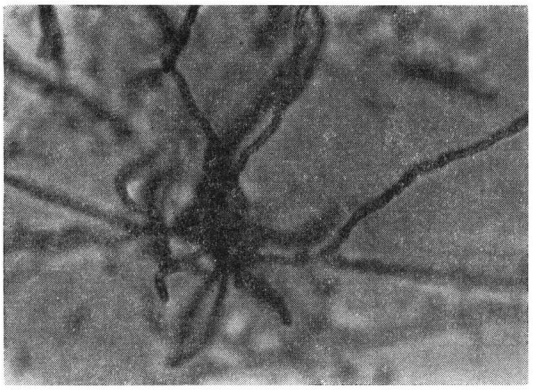
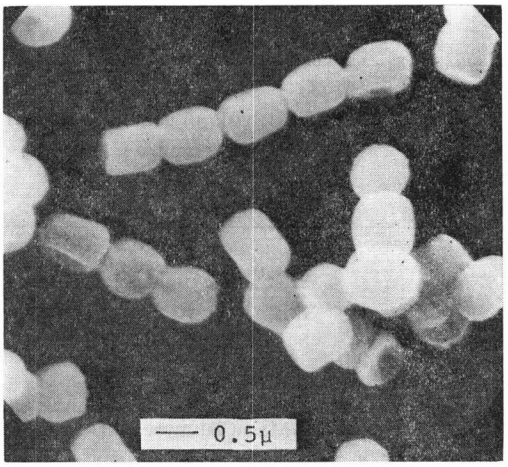


Plate 2. Electron micrograph of spores of strain A271.
Medium: Yeast extract-malt extract agar (14 days).



The surface of the spores is smooth (Plate 2). Neither flagella nor sporangia are observed.

It is therefore believed that this strain belongs to the Section *Retinaculum-Apertum* (RA) of the genus *Streptomyces*.

Cultural Characteristics

Cultural characteristics of strain A271 on media for the taxonomic studies are shown in Table 1. The results were observed after 2-week incubation unless otherwise stated. The color of the aerial mycelium and substrate mycelium was designated mainly on the basis of the seven color series of the color wheels made by TRESNER and BACKUS[13] and also on the color table of "Guide to Color Standard" a manual published by Nippon Shikisai Kenkyusho, Tokyo, Japan.

The aerial mycelium is orange yellow on most media. The substrate mycelium is light yellow or light orange on most media. No diffusible pigment is observed in media commonly used in taxonomic studies.

Physiological Characteristics

The physiological characteristics of the strain are shown in Table 2. Liquefaction of gelatin, hydrolysis of starch, reduction of nitrate and peptonization of milk are positive. Coagulation of milk

Table 1. Cultural characteristics of strain A271.

Media	Cultural characteristics	Media	Cultural characteristics
Oat meal agar	*G: abundant AM: pale orange yellow (3ca)-light orange yellow (3ea) SM: light yellow (2fb) SP: none	Peptone-yeast extract-iron agar	G: abundant AM: absent SM: pale yellow (2db) SP: none
Glycerol-asparagine agar	G: abundant, hygroscopic AM: pale orange yellow (3ca)-light orange yellow (3ea) SM: light yellow(2fb)-moderate yellowish pink(4gc) SP: none	Glucose-asparagine agar	G: abundant AM: pale orange yellow (3ea)-light orange yellow(3ea) SM: light yellow (11/2fb-2fb) SP: none
Yeast extract-malt extract agar	G: abundant AM: pale orange yellow(3ca)-light organge yellow(3ea) SM: light orange yellow(3ea)-pale brown(4ie) SP: none	Nutrient agar	G: moderate AM: hardly formed, if formed somewhat dark SM: pale yellow(2db) SP: none
Inorganic salts-starch agar	G: abundant AM: pale organge yellow(3ca) SM: light orange yellow(3ea) SP: none	Sucrose-nitrate agar	G: moderate AM: light orange yellow(3ea) SM: light yellow(2fb)-light orange yellow(3ea) SP: none
Tyrosine agar	G: abundant AM: light orange(3ea)-yellow SM: light yellow(2fb), later light orange yellow(3ea)-brownish yellow SP: none-brown (trace)	Calcium malate agar	G: moderate AM: pale orange yellow(3ea) SM: light yellow(11/2fb) SP: none

* G: growth, AM: aerial mycelium, SM: substrate mycelium, SP: soluble pigment.
( ): Color code of the Color Harmony Manual[12].

Table 2. Physiological properties of strain A271.

Temperature	growth occurs at 10~40°C better growth at 20~30°C
pH	growth occurs at pH 5~9 optimum at pH 6~8
Liquefaction of gelatin	positive (glucose-peptone-gelatin medium)
Hydrolysis of starch	positive
Milk coagulation	negative
Reduction of nitrate	positive
Production of melanoid pigment	negative (tyrosine agar, peptone-yeast extract-iron agar and tryptone-yeast extract broth)

Utilization of carbon sources by strain A271

Carbon sources	Growth	Carbon sources	Growth
D-Glucose	+	Sucrose	±
D-Fructose	−	Raffinose	−
D-Xylose	+	*i*-Inositol	−
L-Arabinose	+	Cellulose	−
L-Rhamnose	+	Control	−
D-Mannitol	−		

+: good growth
±: poor growth
−: no growth

and formation of melanoid are negative.

L-Arabinose, D-xylose, D-glucose and L-rhamnose are utilized for growth. On the other hand,

D-fructose, *i*-inositol, raffinose and D-mannitol are not utilized for growth. Sucrose is slightly utilized for growth.

Comparison of Strain A271 with Other Known *Streptomyces* Species

From the above characteristics, strain A271 is thought to belong to the genus *Streptomyces* having the following characteristics: Color of mature sporulated aerial mycelium is in the Red color-series; spore chain morphology showing predominantly hooks, loops or incomplete spirals is in Section RA; spore surface is smooth; formation of melanoid pigment is negative.

As species which taxonomically resemble strain A271, the following seven species were selected; *S. cremeus* ISP 5147, *S. flavidovirens* ISP 5150, *S. albohelvatus* ISP 5410, *A. flavescens* ISP 5203, *S. rutgersensis* ISP 5077, *S. chryseus* ISP 5420 and *S. helvaticus* ISP 5431.

Standard strains of the above seven species were compared with strain A271 under the same culture conditions. From the results, all strains except *S. cremeus* differed clearly from strain A271. Tables 3 ~ 5 give the results of the comparison of strain A271 with *S. cremeus* ISP 5147. The following differences were noted.

Color of the aerial mycelium: *S. cremeus* shows a weaker reddish tone than strain A271.

Color of substrate mycelium: *S. cremeus* shows a light orange yellow color in most media while strain A271 generally shows a light yellow color.

Strain A271 differs from *S. cremeus* in the utilization of D-fructose and L-rhamnose.

From the results of the above taxonomic studies strain A271 was considered to be a subspecies of

Table 3. Comparison of strain A271 with *Streptomyces cremeus* ISP 5147.

Color of aerial mycelium

Media	Strain A271	*S. cremeus* ISP 5147
Sucrose-nitrate agar	light orange yellow (3ea)	aerial mycelium hardly formed
Glucose-asparagine agar	pale orange yellow (3ca) to light orange yellow (3ea)	pale orange yellow (3ca)
Glycerol-asparagine agar	pale orange yellow (3ca) to light orange yellow (3ea)	pale orange yellow (3ca)
Inorganic salts-starch agar	pale orange yellow (3ca) to light orange yellow (3ea)	pale orange yellow (3ca)
Nutrient agar	hardly formed, if formed somewhat dark	white (a) to pale orange yellow (3ca)
Yeast extract-malt extract agar	pale orange yellow (3ca) to light orange yellow (3ea)	pale yellow (2db)
Oat meal agar	pale orange yellow (3ca) to light orange yellow (3ea)	white (b) to pale orange yellow (3ca)

( ): color code of the Color Harmony Manual.

Table 4. Comparison of strain A271 with *Streptomyces cremeus* ISP 5147.

Color of substrate mycelium.

Media	Strain A271	*S. cremeus* ISP 5147
Sucrose-nitrate agar	light yellow (2fb) to light orange yellow (3ea)	poor growth colorless to white (b)
Glucose-asparagine agar	light yellow (11/2fb-2fb)	light orange yellow (3ea)
Glycerol-asparagine agar	light yellow (2fb) to somewhat yellowish pink (4gc)	light orange yellow (3ea)
Inorganic salts-starch agar	light orange yellow (3ea) to light yellow (2fb)	moderate yellowish pink (4ea)
Tyrosine agar	light orange yellow (3ea)	pale brown (4ie)
Nutrient agar	pale yellow (2db)	light orange yellow (3ea)
Yeast extract-malt extract agar	light orange yellow (3ea) to pale brown (4ie)	light orange yellow (3ea) to dark yellow
Oat meal agar	light yellow (2fb)	somewhat yellowish pink (4gc)

( ): Color code of the Color Harmony Manual.

S. cremeus and named *S. cremeus* subsp. *auratilis* KOUNO *et* ISHIKURA, based on the color tone on commonly used agar media. A culture of the strain A271 has been deposited in the American Type Culture Collection and assigned accession number ATCC 31358.

Table 5. Comparison of strain A271 with *Streptomyces cremeus* ISP 5147.

Utilization of carbon sources

Carbon sources	Strain A271	*S. cremeus* ISP 5147
L-Arabinose	+*	+
D-Xylose	+	+
D-Glucose	+	+
D-Fructose	−	+
Sucrose	±	−
i-Inositol	−	−
L-Rhamnose	+	−
Raffinose	−	−
D-Mannitol	−	−
Cellulose	−	−

* +: good growth, ±: poor growth, −: no growth.

Disc Plate Assay of PS-5

Comamonas terrigena B-996 isolated from *Comamonas terrigena* IFO 12685 as a strain made especially sensitive to cephalothin was used. Potency of PS-5 in culture broth and in samples during the purification process was expressed using *Comamonas*-Cephaloridine-Unit (CCU). Dose-response curve of cephaloridine and PS-5 on a *Comamonas terrigena* B-996 plate showed good parallelism.

A potency of PS-5 solution which gives the same diameter of inhibition zone on a *Comamonas terrigena* B-996 plate as 100 μg/ml of cephaloridine is expressed as 100 CCU/ml. Ten μg/ml of PS-5 sodium and 210 μg/ml of cephaloridine gave the same size of the inhibition zone. Thus, 1 mg of PS-5 sodium corresponded to 21,000 CCU.

Fermentation

A 30-liter jar fermentor containing 15 liters of seed medium (SE-4, Table 6) was inoculated with 100 ml of a seed culture grown in 500-ml Erlenmeyer flasks on a rotary shaker for 48 hours. The jar fermentor was operated at 28°C using an agitation rate of 200 rpm and an air flow rate of 0.5 vol/vol/min. for 24 hours. One liter of the seed culture was used to inoculate a 200-liter stainless steel fermentor containing 100 liters of the production medium (ML-19M2, Table 6). This fermentor was operated at 28°C using an agitation rate of 100 rpm and an air flow rate of 0.5 vol/vol/min. for 72 hours. The production of PS-5 was followed by a disc plate assay with *Comamonas terrigena* B-996 as the assay organism. Growth was measured using the packed volume of sediment from 3 ml of broth after

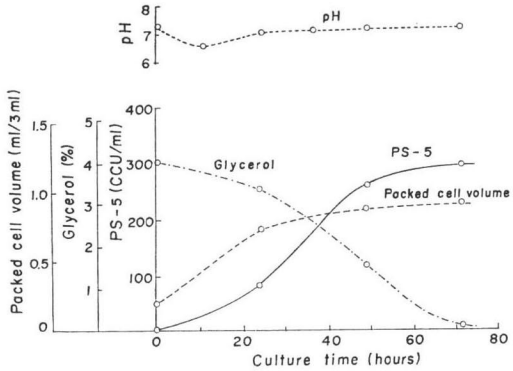
Table 6. Seed medium and fermentation medium.

Seed medium (SE-4)		Production medium (ML-19M2)	
Beef extract (Difco)	0.3%	Glycerol	4.0%
Bacto-tryptone (Difco)	0.5	Peptone	0.5
Defatted soybean meal	0.5	Glucose	0.2
Glucose	0.1	Potato starch	0.2
Sol. starch	2.4	Defatted soybean meal	2.5
Yeast extract	0.5	Dry yeast	0.5
$CaCO_3$	0.4	NaCl	0.5
	pH 7.5	$CaCO_3$	0.2
			pH 6.4

centrifugation at 1,500 *g* for 10 minutes. Glycerol was determined by the method of IWAI *et al.*[14].

Paper chromatography and thin-layer chromatography of the broth filtrate gave only single bioactive spot, but when the concentrated eluate from HP20 resin was subjected to the chromatography, very minor components were detected, therefore, the titre of PS-5 in broth was expressed in CCU/ml in Fig. 1. The time course of a PS-5 fermentation is shown in Fig. 1.

Fig. 1. Time course of PS-5 fermentation in a 200-liter fermentor
Medium: ML-19M2, Temperature: 28°C.



Isolation

A schematic representation of the isolation process is shown in Fig. 2.

Perlite (filter aid) was added to the whole broth and the culture broth filtered. The clarified broth was passed through Diaion PA306 ion-exchange resin (Cl^- form) followed by adsorption on Diaion HP20 resin and eluted with 75% methanol. The active fraction was diluted with three volumes of deionized water and applied on a Diaion PA306S ion-exchange column. After washing with water, the antibiotic was eluted from the resin with 0~3% NaCl (gradient). The eluate was applied to a Diaion HP20 resin column and eluted with a linear gradient of acetone (0~25%). After removing acetone *in vacuo*, the eluate was adsorbed onto a QAE-Sephadex A-25 column and eluted with a linear gradient of NaCl (0~1.5%). The eluate (pH 8.3) was applied to a Diaion HP20AG column and eluted with a linear gradient of acetone (0~10%). The active fraction was lyophilized and yielded 249 mg of yellow powder (8,000 CCU/mg).

One hundred and fifty mg of the crude powder was dissolved in a small amount of M/100 sodium phosphate buffer (pH 8.0) and purified by Sephadex G-10 gel filtration. The active fraction was then applied to a QAE-Sephadex A-25 column and eluted with linear gradient of NaCl (0~1.5%). The eluate (pH 8.3) was applied to a Diaion HP20 column and eluted with linear gradient of acetone (0~10%). After lyophilization of the active fraction, 51 mg of a white powder was obtained (20,000 CCU/mg).

Physico-chemical Properties of PS-5

The physico-chemical properties of PS-5 are shown in Table 7. PS-5 sodium salt is soluble in water and substantially insoluble in acetone, ethyl acetate and benzene. PS-5 did not show a clear melting point and turned yellow around 148°C and gradually decomposed above 160°C when measured in a Kofler apparatus BY-1 (Yazawa Scientific Mfg. Co., Ltd.) The PS-5 showed a UV maximal absorption at 301 nm and a minimal at 246 nm (in H_2O). The IR spectrum of PS-5 sodium salt taken in KBr is shown in Fig. 3. Characteristic absorptions attributable to β-lactam, amide and carboxylate were seen at 1760, 1550 (1650) and 1600 cm^{-1}, respectively. NMR spectrum were recorded at 100 MHz using 3-(trimethylsilyl) propionic acid-d_4-sodium salt as the internal reference (Fig. 4). There were signals at δ 1.06 (3H, t, $\underline{CH_3}$-CH_2-), 1.72~2.00 (2H, CH_3-$\underline{CH_2}$-), 2.05 (3H, s, $\underline{CH_3}$-CO-), 2.88~3.58 (7H, -CH_2-, -$\underline{\overset{|}{N}}\underline{H}$-) and 4.04 ppm (1H, dt, J=3.0, 9.2 Hz, -$\underline{\overset{|}{N}}$H-).

Fig. 2. Isolation of PS-5.

Broth
↓ Perlite 5%
Broth filtrate, 160 liters 235 CCU/ml
↓
Diaion PA306 (4.5 liters)*
↓
Diaion HP20 (15 liters)
↓ elution with 75% MeOH
Active fraction, 2 liters
↓
Diaion PA306S (2 liters)
↓ gradient elution with 0~3% NaCl soln.
Active fraction
↓
Diaion HP20 (1 liter)
↓ gradient elution with 0~25% aqueous acetone
Active fraction, 390 ml 27,220 CCU/ml
↓
QAE-Sephadex A-25 (200 ml)
↓ gradient elution with 0~1.5% NaCl soln.
Active fraction 140 ml 42,120 CCU/ml
↓
Diaion HP20AG (200 ml)
↓ gradient elution with 0~10% aqueous acetone
Active fraction, 60 ml 45,000 CCU/ml
↓
Freeze dry
↓
Crude powder, 249 mg 8,000 CCU/mg
↓
Crude powder, 150 mg dissolved in PBS
↓
Sephadex G-10 (130 ml)
↓
Active fraction
↓
QAE-Sephadex A-25 (100 ml)
↓ gradient elution with 0~1.5% NaCl soln.
Active fraction, 50 ml 22,000 CCU/ml
↓
Diaion HP20AG (50 ml)
↓ gradient elution with 0~10% aqueous acetone
Active fraction
↓
Freeze-dry
↓
White powder, 51 mg 95% pure

* Column bed volume in parenthesis.

Table 7. Physico-chemical properties of PS-5.

Appearance	white powder
Solubility	soluble: water insoluble: acetone, ethyl acetate, benzene
m.p.	turns yellow around 148°C, decomp. above 160°C
UV nm ($E^{1\%}_{1cm}$, H_2O)	λ_{max} 301 (267.5), λ_{min} 246 (82.0) shows a hydroxylamine extinguishable absorbance of 94% (301 nm)
IR (KBr) cm^{-1}	1760, 1650, 1600, 1555, 1400
PMR ppm (100 MHz, D_2O)	1.06 (3H, t, J=7.0 Hz), 1.72~2.0 (2H), 2.05 (3H, s), 2.88~3.58 (7H), 4.04 (1H, dt, J=3.0, 9.2 Hz)
$[\alpha]_D^{22}$	+1.23 (*c* 1.59, 0.01 M, pH 8, PBS)
Mass spectrum (Methyl ester)	312.1131 (M^+ of the methyl ester) 312.1143 (calcd. $C_{14}H_{20}N_2O_4S$)
Mol. formula (Sodium salt)	$C_{13}H_{17}N_2O_4SNa$
Color reaction	positive: EHRLICH reagent, iodine-chloroplatinic acid negative: ninhydrin
PPC (descending)	*n*-propanol - water (7: 3) Rf=0.68
	n-propanol - isopropanol - water (7: 7: 6) Rf=0.70
	acetonitrile - water (8: 2) Rf=0.36
	acetonitrile - tris buffer - EDTA* Rf=0.34
	ethanol - water (7: 3) Rf=0.63
TLC	Silica gel F_{254}, ethanol - water (7: 3) Rf=0.82
	Avicel cellulose, isopropanol - water (7: 3) Rf=0.96
Paper electrophoresis	migration distance to anode (pH 8.6 Veronal buffer, I=0.05, 42V/cm, 30 min.): 28 mm

* acetonitrile, 120 ml: M/10, pH 7.5 tris buffer, 30 ml: M/10, pH 7.5 EDTA, 1 ml.

phic behaviors are shown in Table 7.

mass spectrum of the methyl ester of PS-5, the molecular ion peak was observed at *m/e* 312.1131 (M^+, 312.1143 calcd. for $C_{14}H_{20}N_2O_4S$). Other physico-chemical properties and chromatogra-

Experimental

General methodology

The UV absorption spectrum was taken with a Hitachi 200–20 spectrophotometer; IR absorption with a Hitachi 260–30 spectrophotometer; optical rotation values with a JASCO DIP-181 digital polarimeter; high resolution mass spectrum with a Hitachi RMU-7 mass spectrometer.

Fig. 3. IR Spectrum of PS-5 sodium salt (KBr)

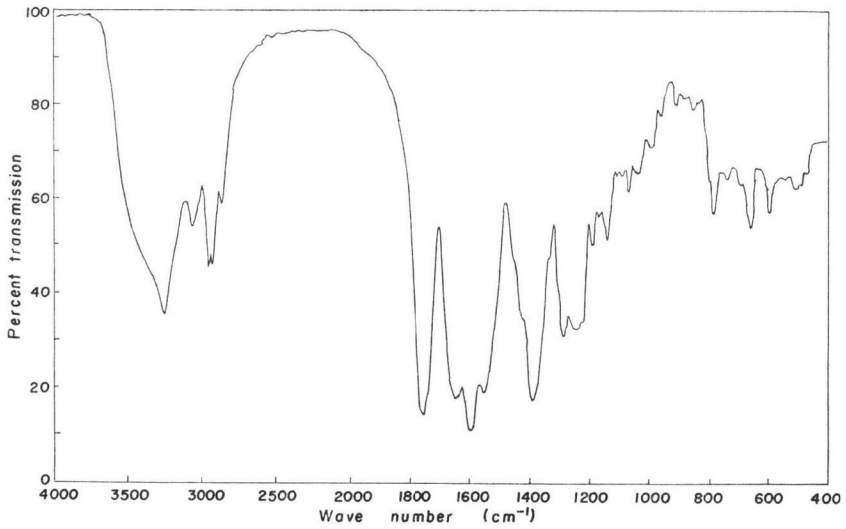
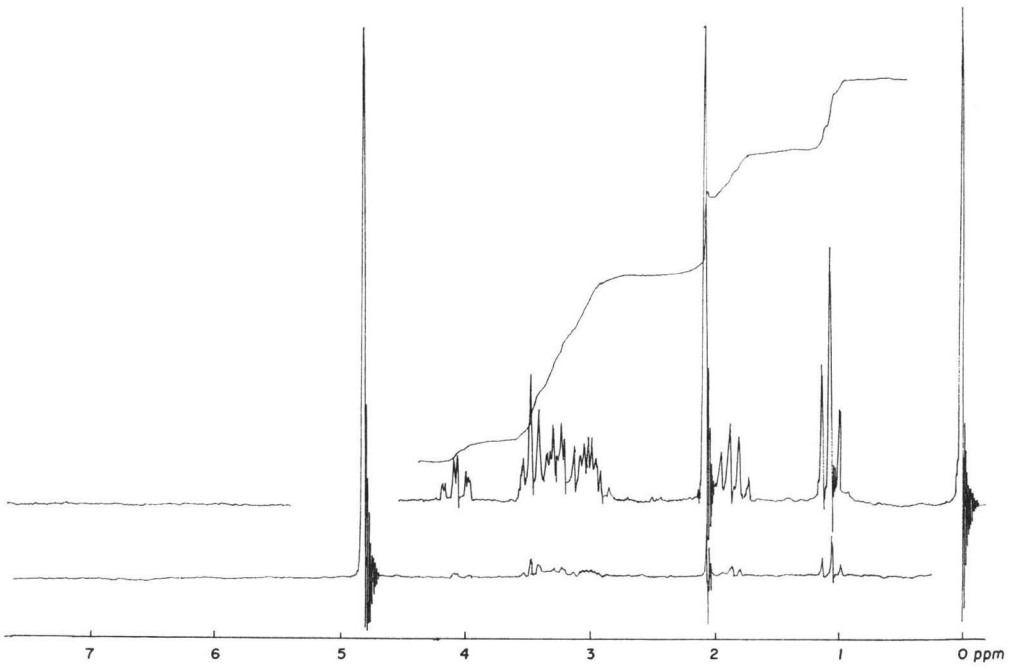


Fig. 4. NMR Spectrum of PS-5 sodium salt (D_2O, 100 MHz)



Methyl ester of PS-5

Fifty mg of triethylamine and 0.3 ml of methyl iodide were added to a suspension of PS-5 sodium salt (30 mg) in 3 ml of dried dimethylformamide. The suspension was stirred for 2.5 hours at room temperature and benzene was added to the suspension to a volume of 100 ml.

The solution was washed with 100 ml of sodium phosphate buffer solution (0.1 M, pH 6.8) and dehydrated with Na_2SO_4. The solution was concentrated to a small volume under reduced

pressure, applied to a Biobeads S-X3 column and eluted with benzene. The eluted ester was chromatographed on a Sephadex LH-20 column with acetone-elution. After drying under reduced pressure, 11.2 mg of PS-5 methyl ester was obtained.

Discussion

Streptomyces cremeus was reported to produce an antibiotic cremeomycin[15]. Since the antibiotic cremeomycin is not a β-lactam antibiotic, this paper is the first description that a strain belonging to *S. cremeus* produced a β-lactam antibiotic. As described in a separate paper[16], in the course of characterization study on PS-5 by the biological properties using assay organisms, *Comamonas terrigena* B-996 and B-996R which are sensitive and resistant to cephalothin respectively and β-lactamases of *Citrobacter freundii* E-9 and *Bacillus licheniformis*, the antibiotic PS-5 was suggested to be a β-lactam antibiotic differentiated from the known penicillins and cephalosporins including 7-methoxy cephalosporins.

Recently a new type of β-lactam antibiotic has been discovered which includes olivanic acid derivatives (MM 4550[17], MC696-SY2-A[18], MM 13902[17] and MM 17880[19]), thienamycin[20] and its derivatives (N-acetylthienamycin[21] and epithienamycin[22]). PS-5 was considered to be a member of these new types of β-lactam antibiotic from its biological and physico-chemical properties. Some distinct differences between PS-5 and these new types of β-lactam antibiotics are mentioned below.

PS-5 was distinguishable from olivanic acid derivatives by a lack of O-sulfate suggested from IR spectrum, high voltage electrophoresis and sulfur content in the molecule. PS-5 was also distinguishable from thienamycin by the behavior on high voltage paper electrophoresis and color reaction with ninhydrin. In addition, PS-5 was differentiated from all the above new type β-lactam antibiotics by signals in the high field (1~2 ppm) of ^{1}H-NMR spectrum. Namely, PS-5 gave a triplet at around δ 1 ppm, while the others gave a doublet at δ 1.2~2.0 ppm. Thus, PS-5 was considered to be a novel β-lactam antibiotic.

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